THE HAIRY ROOT CULTURE OF *POLYGONUM TINCHTORIUM* AIT FOR ANTHOCYANIN PRODUCTION

KULTUR AKAR DARI *POLYGONUM TINCHTORIUM* AIT UNTUK PRODUKSI ANTOSIANIN

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ABSTRACT

The hairy root culture of Polygonum tinchtorium Ait was established through infection of sterilized seedling with bacteria Agrobacterium rhizogenes strain A_4 and ATCC 15834. There were variation in anthocyanin production among isolated clones. The anthocyanin produced by hairy root was smaller than produced by plants in field. Although agropine and mannopine spots were undetectable by paper electrophoresis, the hairy root cultures was proved to be transformed by bioassay.

RINGKASAN

Kultur akar transgenik dari *Polygonum* tinchtorium Ait telah dimantapkan dengan cara menginfeksi bibit steril tanaman ini dengan bakteri *Agrobacterium rhizogenes* strain A_4 dan ATCC 15834.

Terdapat variasi kandungan antosianin diantara klon yang diisolasi. Kandungan antosianin yang dihasilkan oleh akar transgenik lebih rendah daripada tanaman yang ada di lapang. Meskipun spot agropine dan mannopine tidak dapat dideteksi dengan elektroforesis kertas, tetapi akar transgenik tersebut telah terbukti bersifat transformasi dengan metode bioassay.

INTRODUCTION

The previous study on the production of anthocyanin from callus culture and suspension culture of *Polygonum tinchtorium* Ait showed that the highest anthocyanin production was achieved in suspension cultures. The selected clone through selection in solid medium produced anthocyanin four times higher than unselected clone. By using suspension cultures, anthocyanin production was ten times higher. (**Ernawati** *et al*, 1991 [a, b]). After some passages of suspension cultures, some problems arised, like unstability clone in producing anthocyanin, which then the repeated slection was necessary to maintain its genetic stability. The development of stabil and fast growing of hairy root cultures may overcome those problems.

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The hairy root cultures were obtained by genetic transformation of plant tissue with bacteria *Agrobacterium rhizogenes*. Its infection on dicotyledon plants caused roots proliferation on the site of infection. It happened because Ri plasmid carried T-DNA of the bacteria was inserted to plant genome, which then induced auxin synthesis and other rhizogenic functions. The induced roots can be isolated from the host plant and cultured on the medium without plant growth regulators. In this case, the hairy root culture is different from the usual root culture which need exogenous auxin for its prolieration. The hairy root cultures has been reported successfully in producction of useful compounds from various plants. (**Ko** *et al*, 1988; **Isa** *et al*, 1989; **Taya** *et al*, 1989). Here, we report the used of hairy root culture of *Polygonum tinchtorium* Ait to produce antosianin.

MATERIAL AND METHODS

Material : Seeds of *Polygonum tinchtorium* Ait cv. Miyagi Ai. Strain bacteria *A. rhizogenes* which contained plasmid pRi A_4 and pRi 15834.

Methods :

1. Initiation of the hairy root culture

The seeds were sterilized with NaHC10 5% and sterilized water, then placed on the medium **Murashige-Skoog** without any plant growth regulator (MS-0). The cultures were then incubated under continuous 3000 lux white lamp and 25°C. (standart condition). The bacteria was then inoculated on the wounded stem of ten days old seedlings. The hairy roots were appeared about two weeks after inoculation. The hairy roots which has 10 cm long, was transferred to the MS-0 medium which contained 500 ppm anti bacteria Clarofuran in petri dish. The cultures were placed on the standart condition for two weeks and subcultured for three times. After that, the hairy root was propagated on liquid MS-0 medium which incubated in standart medium and sub cultured every two weeks.

2. The exploration of Hairy root clones

Each hairy root which isolated from one seedling was placed on a petri dish which contained solid MS-0 medium. Each petri dish contained 2 pieces 2 cm long hairy root. Observation was done for the growth and dry weight, and anthocyanin synthesis which showed by red colour. The red colour hairy root was then transferred to liquid MS medium. The explants were 5 cm of root tip with branches. There 5 bottles for each clone. The observation was done for anthocyanin synthesis and dry weight after culture 1 month old.

3. Detection of transformed hairy root

Detection of agropine and mannopine was carried out according **Petit** *et al* (1983). One month old hairy root was used as material. 1 gram fresh weight of hairy root was macerated at 4°C for 10 minutes, then 0.5 ml HCl 1N was added. This solution was then poured into ependorf tubes dan centrifuged at 14 000 rpm at 4°C for 10 minutes. The supernatan was then charged on the filter paper, and the volume was 50 ul, 100 ul, 150 ul, 200 ul and 250 ul. The supernatant of transformed hairy root of tomato was used as standart. The methylene blue was used as marker.

Running buffer : Buffer solution was containing 25 ml formic acid, 75 ml acetic acid and 400 ml destilated water. Electrophoresis was done at CV 400 volt for 3 hours. After that the paper was taken out and dried up at room temperature. The paper then was soaked in buffer solution A for 8 minutes, for staining. The buffer solution A containing 6 gram $AgN0_3$ soluted in 0.6 ml destilated water and 300 ml aceton. After that, the paper was removed to buffer solution B for three minutes, which contained 160 ml of 20% NaOH solution and 160 ml ethanol. The paper then was rewashed in distiled water dan then was soaked in fuji fix solution for 2-3 hours. After that the paper was dried up.

Bioassay to prove the hairy root was transformed or untransformed

Each hairy root clone was out into 0.5 - 1 cm long and placed in solid MS medium which contained 10^{-6} M and 10^{-5} M and incubated at standart condition. The calli formed in those medium was then removed and transferred to liquid MS-0 medium and incubated at standart condition, and shaked at 125 rpm.

4. The effect of explants, sucrose and NH_4 NO_3 on hairy root growth and anthocyanin synthesis

The explant was from 2 weeks old culture stock. There were 3 type of explants: (a) 5 cm long root tip with branches, (b) 5 cm long root tip without branches, (c) a piece of explant type a and b together placed in one bottle.

The sucrose concentration was 0; 15 and 30 g/l. The NH_4 NO_3 concentration was 0, 2, 20 m. Basal medium was Murashige-Skoog. There were 5 bottles for each treatment.

Observation was done for the growth and development of hairy root, anthocyanin synthesis and dry weight. The anthocyanin measurement was done according **Ernawati** *et al* (1991).

RESULT AND DISCUSSION

1. Initiation of hairy root culture

The hairy root was showed up about 2 weeks after inoculation of bacteria on the wounded stem of sterilized seedling. The percentage of hairy root formation after infection by *A. rhizogenes* strain A_4 and ATCC 15834 was same (80%). The effectivity of both strains was same to induce hairy root.

2. Exploration of hairy root clones on solid and liquid medium

The preliminary growth of the hairy root was slow. After 3 weeks old, the maximum root was 15 cm with lateral branches. Total isolated clones was 23 clones hairy root, initiated with *A. rhizogenes* strain A_4 and ATCC 15834. But only some clones produced anthocyanin. This result may be because the initiation of hairy root was through seedlings which has different genetic potential. The capacity of hairy root to produce secondary metabolite was as same as the mother plant's (**Flores** and **Filner**, 1985). According to **Cardarelli** *et al* (1985) the root proliferation by *A. rhizogenes* was depent on certain host plant and certain organ. **Owens** and **Cress** (1985) reported that each strain has different ability to infect cultivar or

species. The different of source of explants could be the cause of different hairy root potensial.

On the liquid medium, the hairy root grew faster than on the solid medium. There were more lateral branches formed. But red pigment showed on the hairy root grew on the liquid medium. There were variation of anthocynin production and dry weight among isolated clones (Table 1). The anthocyanin content was lower than the one produced by suspension culture, calli and intact plant. It may because anthocyanin was produced in stem and flower of intact plant, while hairy root usually produce compounds which naturally was produced in root of intact plant.

Table 1. The anthocyanin content and dry weight of the *Polygonum tinchtorium* Ait hairy root clones (1 month old)

Clones	*A. rhizogenes* A_4		*A. rhizogenes* 15834	
	Anthocyanin A_{525}	Total Dry weight g	Anthocyanin A_{525}	Total D. weight g
1.	0.186+0.006	0.057+0.009	0.211+0.003	0.107+0.01
2.	0.241+0.03	0.054+0.008	0.127+0.02	0.108+0.02
3.	0.123+0.02	0.092+0.01	0.160+0.01	0.104+0.02
4.	0.124+0.01	0.101+0.01	0.122+0.01	0.122+0.03
5.	0.110+0.02	0.099+0.01	0.125+0.01	0.089+0.009
6.	0.214+0.03	0.114+0.02	0.190+0.02	0.116+0.02
7.	0.180+0.03	0.090+0.01	0.196+0.02	0.078+0.008
8.	0.252+0.02	0.110+0.02	0.161+0.02	0.114+0.02
9.	0.115+0.03	0.105+0.01	0.200+0.03	0.115+0.01
10.	0.2 +0.03	0.101+0.02	0.230+0.03	0.128+0.02
11.	0.130+0.01	0.097+0.009	0.118+0.02	0.078+0.009
12.	0.273+0.03	0.087+0.008		

3. Detection of transformed hairy root

The experiment by using paper electrophoresis was not able to detect specific spot of agropinnic acid and mannopinic acid extracted from the hairy root culture of *Polygonum tinchtorium* Ait. In contrary, the clear spot was detected from transformed tomato's hairy root that was used as marker. The result doesn't mean that the hairy root culture of *Polygonum tinchtorium* Ait was not transformed.

The bioassay experiment showed that the hairy root induced callus in solid MS medium containing 10^{-6} M NAA and 10^{-5} M NAA. Some of calli was friable and some of them was compact. All calli formed hairy root again when those calli were transferred on the liquid MS-0 medium. This phenomena showed that those hairy roots were transformed. Opine is spesific amino acid which used as indicator of transformation. But the expression of the gene codes opine synthesis in hairy root was not stabil along time. (**Rhodes** *et al*, 1987). In the case of hairy root of Nicotiana rustica, opine spot also was not able to be detected by paper electrophoresis, but by using Southern blot hybridization, the existence of T_R-DNA and T_L-DNA of hairy root was detected. (**Rhodes**, *et al*, 1987).

4. The effect of explant, sucrose and NH_4NO_3 on the growth and anthocyanin production of hairy root

The experiment was visually observed and its result was showed on the table 2.

Table 2. The condition of hairy root after 2 months in treatment medium

Sucrose / NH_4NO_3	30 g	15 g	0 g
20mM	a. growing, numerous branches (++++)	growing, numerous branches (++++)	death, no growth
	b. growing, long straight	growing, long straight	death, no growth
	c. growing, numerous branches (++++)	growing, numerous branches (++++)	death, no growth
2 mM	a. growing, few branches (++)	growing, numerous branches(++++)	death, no growth
	b. growing, few branches (++)	growing, numerous branches (++++)	death, no growth
	c. growing, few branches (++)	growing, numerous branches (++++)	death, no growth
0 mM	a. death, no growth	death, no growth	death, no growth
	b. death	death	death,
	c. death	death	death,

a,b,c; type of exsplants
(+) ; showed the visual appearance

Table 2 showed that both of sucrose and NH_4NO_3 were needed by explant to support the growth of hairy root. According to **Bomhoff** *et al* (1976) the inserted Ti plasmid in genome will use the opine as source of energy, as N and C source. The same analogy was also take place for Ri plasmid, which induced agropine and mannopine. The lateral branches from the root was formed from explant type b which placed on the MS medium containing 2 mM NH_4NO_3 at the sucrose concentration 15 gram or 30 gram. This data showed that NH_4NO_3 was critical factor for the growth and development of explant. Sucrose was needed as energy source. The dry weight of hairy root was showed on the Table 3.

Table 3 showed that the highest dry weight was achieved when the explant used was root tip with lateral branch. The condition was MS media containing 2 mM NH_4NO_3 and 15 g sucrose. In case of cell suspension culture of soybean the growth was decreased if the concentration of NH_4NO_3 was higher than 2 mM on the B-5 medium (**Gamborg** *et al*, 1968).

Table 4 showed that the highest anthocyanin was produced by cultures grow on the media containing NH_4NO_3 2 mM, 15 g/l sucrose by using branches root tip as an explant. This result was different from the one take place in suspension cultures. In suspension cultures of *P. tinchtorium* Ait the deletion of NH_4NO_3 from the MS mediumm increased anthocyanin synthesis (**Ernawati** *et al*, 1991). The incubation time of hairy root cultures was 2 months, while the suspension cultures was 2 weeks. During that time, degradation of anthocyanin synthesis may take place. The capacity of the hairy root cultures to produce anthocyanin was lower than the suspension cultures. The production of anthocyanin *in vivo* was take place at stem and flower. In the suspension cultures of *Polygonum tinchtorium* Ait, the cells was able to proliferate on the MS medium without NH_4NO_3 while on the hairy root cultures, explants was dying when those placed on MS medium without NH_4NO_3 while on the hairy root cul-

tures, explants was dying when those placed on MS medium without NH_4NO_3. It is clear that NH_4NO_3 was necessary for the growth of hairy root culture.

Table 3. The dry weight of hairy root after 2 months cultured

Sucrose		30 g	g15 g	0 g
NH_4NO_3				
20mM	a.	0.109+0.002	0.169+0.02	0.008 +
	b.	0.035+0.004	0.052+0.006	0.004
	c.	0.091+0.008	0.14 +0.02	0.010
2mM	a.	0.225+0.03	0.247+0.03	0.008
	b.	0.109+0.01	0.109+0.01	0.004
	c.	0.228+0.02	0.223+0.03	0.010
0mM	a.	0.007	0.006	0.007
	b.	0.002	0.004	0.003
	c.	0.009	0.009	0.010

Table 4. The anthocyanin content of hairy root after 2 months old. (A_{525})

Sucrose NH_4NO_3		30 g	g15 g	0 g
20mM	a.	0.102+0.02	0.167+0.03	0
	b.	0.020+0.008	0.030+0.005	0
	c.	0.080+0.006	0.125+0.02	0
2mM	a.	0.194+0.03	0.238+0.03	0
	b.	0.090+0.006	0.125+0.02	0
	c.	0.201+0.03	0.184+0.02	0
0mM	a.	0	0	0
	b.	0	0	0
	c.	0	0	0

CONCLUSION

The experiment showed that both of *A. rhizoqenes* strains A_4 and ATCC 15834 effectively induced hairy root culture of *P. tinchtorium* Ait. The Hairy Root obtained was transformed root.

The anthocyanin content produced by Hairy Root was little and lower than the one produced by callus cell line and intact plant.

The best condition to support the growth of hairy root and anthocyanin synthesis was MS medium containing 2 mM NH_4NO_3 and 15 g/l sucrose.

DAFTAR PUSTAKA

Bomhoff, G., P.M. Klapwijk, H.C.M. Kester, R.A. Schilperoort, J.P. Hernalsteens, and J. Schell. 1976. Octopine and nopaline synthesis and breakdown genetically controlled by a plasmid of *Agrobacterium tumefaciens*. Mol. Gen. Genet. 145:177-181

Cardarelli, M., Spano, A. De Paolis, M.L. Mauro, G. Vitali, and Constantino, P. 1985, Identification of genetic locus responsible for non polar root induction by *Agrobacterium rhizogenese* Ri plasmids. Plant Moll. Biol. 6, 271-279

Ernawati, A., S. Mayama and M. Kyo. 1991. Selection of high yielding anthocyanin callus line of *Polygonum tinchtorium* Ait. Buletin Agronomi 20 (1): 1-7

Ernawati, A., M. Kyo and S. Mayama. 1991. Study on the induction of anthocyanin synthesis in suspension cultures of *Polygonum tinchtorium* Ait. Indonesia Journal of Tropical Agriculture 2 (2) : 73-79

Flores, H.E. and P. Filner. 1985. Metabolic relationships of putrescine, GABA and Alkaloids and cell and root cultures of solanaceae. *In* Neumann K.H., Barz, W. and Reinhard, E. (Eds.). Primary and Secondary metabolism. p. 174-185. Springer-Verlag, Berlin and Heidelberg.

Gamborg, O.L., R.A. Miller, K. Ojima. 1968. Nutrient require ments of suspension cultures of soybean root cells. Exp. Cell. Res. 50, 151-158

Isa, T., T. Ogasawara, and H. Kaneko, 1989. Induction of hairy root from Astragalus plant, and mucilage production by the hairy root. Plant Tissue Culture Letters. 6(3) : 134-137

Ko, Kyung Soo, Y. Ebizuka, H. Noguchi and U. Sankawa. 1988. Production of secondary metabolits by hairy roots and regenerated plants transformed with Ri plasmid, Chem. Pharm. Bull. 36(10) : 4217-4220

Owens, L.D., Cress, D.E. 1985. Genotypic variability of soybean response to *Agrobacterium* strains harboring Ti or Ri plasmids. Plant Physiol. 77 : 87-94

Petit, A., C. David, G.A. Dahl. J.G. Ellis, P. Guyon, P. 1983. Further extension of the opine concept: plasmids in Agrobacterium rhizogenes cooperate for opine degradation. Mol. Gen. Genet. 190: 204-214

Rhodes, M.J.C., R.J. Robins, J.D. Hamill, A.J. Parr and J.N. Walton. 1987. Secondary product formation using Agrobacterium rhizogenes-transfom mediary Root cultures. Newsletter of International Association for Plant Tissue Culture p. 2-5

Taya, M., A. Yayama, O. Kondo, H. Honda and T. Kobayashi. 1989. Hairy-Root from Pak-Bung for peroxidase production. Plant Tissue Culture Letters. 6(3): 159-161.